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SARCOMERE LENGTH–TENSION RELATIONS OF FROG SKINNED MUSCLE FIBRES AT LENGTHS ABOVE THE OPTIMUM

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SUMMARY

1. Single twitch fibres were dissected from anterior tibial muscles of the frog, *Rana pipiens*, and were then either chemically or mechanically skinned. Short segments of the skinned fibres were transferred to an experimental chamber and mounted between a force transducer and a stationary wire.

2. The average sarcomere length was determined from light photomicrographs of the segments obtained during activation and while relaxed. Activations were maximal, in solutions of pCa 5·49 and at 5 °C. Fibre segments having regions in which the striation pattern was highly non-uniform were rejected.

3. The relationship between tension and average sarcomere length was determined for sarcomere lengths between 2·1 and 3·8 μm. Tension always decreased when sarcomere length was increased above about 2·2 μm. Tension plotted against over-all average sarcomere length lay above data obtained from living fibres by Gordon, Huxley & Julian (1966*a*, *b*).

4. Good agreement with living fibre results was found when tension was plotted against the shortest average sarcomere length within a portion of the segment.

5. These findings indicate that sarcomere length non-uniformities greatly influence the shape of the sarcomere length–tension relation in skinned fibres at long lengths. In addition, no evidence was found for a length-dependent influence of calcium on tension development at long lengths during maximal activation.

INTRODUCTION

The relationship between isometric tetanic tension and sarcomere length in frog skeletal muscle fibres has been described by Gordon *et al.* (1966*a*, *b*). Between sarcomere lengths of about 2·05 and 2·25 μm, there is a tension plateau the width of which approximately equals the length of the bare zone on the thick filaments. Above 2·25 μm, which will be called the descending limb of the length–tension relation, tension declines linearly as sarcomere length is increased, corresponding to a decreased amount of thick and thin filament overlap, reaching zero at a sarcomere length of about 3·65 μm. Deviations from this inverse linear relationship in living, tetanically stimulated fibres can be explained on the basis of sarcomere

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length non-uniformities along the fibres (Huxley & Peachey, 1961; Julian, Sollins & Moss, 1978; Julian & Morgan, 1979). Studies (Hellam & Podolsky, 1969; Schoenberg & Podolsky, 1972) of the descending limbs of skinned muscle fibres have yielded results similar to those of Gordon *et al.* (1966*a*, *b*).

Despite the agreement obtained in these studies on living and skinned fibres, there has been much recent discussion regarding the interpretation and the actual shape of the descending limb. For example, Iwazumi & Pollack (1979) found isometric tension in skinned fibres to actually *increase* at lengths above 2·2 μm. None of the previous studies on skinned fibres has attempted to measure striation spacing microscopically from end to end of the fibre segments during contraction. The present study further investigates the descending limb in segments from skinned fibres that are maximally activated with calcium, paying particular attention to striation spacing along the whole of the segments. The fibre segments were observed and photographed through a microscope, allowing direct measurement of sarcomere length and also an analysis of sarcomere length uniformity during activation. The results obtained at maximal activation substantially agree with those of Gordon *et. al.* (1966*a*, *b*) when local sarcomere length non-uniformities of the skinned fibres are taken into account. Some aspects of this work have been presented at a meeting of the (American) Biophysical Society (Moss & Julian, 1978).

METHODS

Preparation

Dissection. Single twitch fibres were isolated from the medial heads of anterior tibial muscles of the frog, *Rana pipiens*, using 27-gauge hypodermic needles and knives broken from razor blades. The dissection was done in Ringer's solution composed of the following (mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85; pH 7·0. Frogs were obtained during both the summer and winter months and were stored in the cold (about 6 °C) for a period of up to 3 weeks before use.

Skinning techniques. The isolated living fibres were extended to the length at which they were just taut, corresponding to a sarcomere length of 2·0–2·1 μm. The fibres were then depolarized in 'high potassium solution' (Julian, 1971) containing (mM): KCl, 100; EGTA, 2; phosphate buffer, 10; pH 7·0. Some of the fibres were then chemically skinned, using the solutions and procedures of Julian (1971). The remainder of the fibres were mechanically skinned (Natori, 1954) using the bent tips of hypodermic needles to roll back the sarcolemma. Mechanical skinning was done in 'relaxing solution' (Julian, 1971), which contained (mM): KCl, 100; $MgCl_2$, 1; ATP, 4; EGTA, 2; imidazole 10; pH 7·0. Care was taken during this procedure to prevent excessive stretching of the freshly skinned part of the fibre.

Transfer and attachment. Segments approximately 3 mm long were cut from the skinned fibres for transfer to the experimental chamber. The chemically skinned segments were transferred in high potassium solution, and the mechanically skinned segments in relaxing solution. The segments were placed horizontally between a force transducer and a wire firmly secured in the chamber. Attachment to the segment ends was done with connectors that have been described previously (Moss, 1979). A portion of the segments, 0·6–1·2 mm in length, remained exposed to the solution between the connectors.

Activating solutions. The free calcium concentration of the activating solutions was controlled using EGTA, or ethlenebis (oxyethylenenitrilo)-tetraacetic acid, with an apparent stability constant for the Ca-EGTA complex of $10^{6·68}$, as described by Julian (1971). The relationship between steady tension and pCa is shown for four segments in Fig. 1. The experiments were done using a saturating level of calcium, pCa 5·49. All tension measurements were done at 5 °C.

Apparatus and procedures

The experimental apparatus and special techniques of procedure have been described in detail elsewhere (Moss, 1979); therefore, the following only briefly describes these aspects of the methods.

Experimental chamber. This consisted of an aluminum plate containing a trough which was divided with Plexiglas (Rohm & Haas) into three compartments. All exposed aluminium surfaces

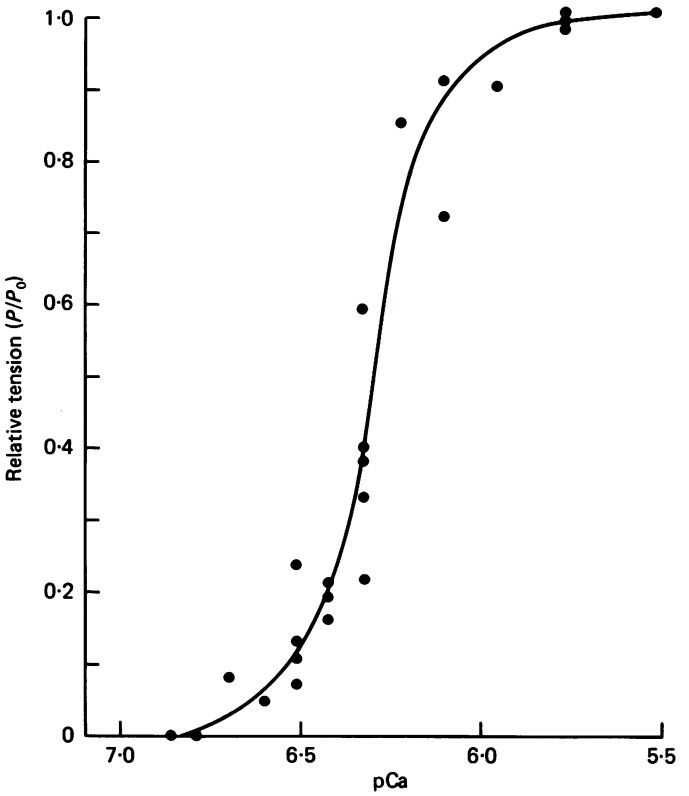

Fig. 1. Plot of developed tension *vs.* pCa (i.e., negative log of free calcium concentration) for four chemically skinned segments. The average sarcomere length during contraction was between 2·10 and 2·22 μm. Tensions are expressed relative to the tension developed by each segment at pCa 5·49. The curve was fitted to the data points by eye.

were sprayed with Teflon (DuPont Co.), and the floor was made of Plexiglas to permit illumination from below for microscopy. Any of the three compartments could be placed beneath the fibre segments by depression and lateral sliding of the plate. The force transducer and fixed wire were mounted on three-way translators for positioning the segments. Cooling of the plate was done with thermoelectric devices. The entire chamber assembly was placed on the modified stage of a Zeiss WL microscope.

Force tranducer. The force transducer was a capacitance gauge type (Julian & Sollins, 1973), with a sensitivity of 1 mV/mg, resonant frequency of about 800 Hz and compliance of 4·7 μm/g. Capacitance changes were detected using a phase sensitive circuit (Cambridge & Haines, 1959), the output of which was recorded on a storage oscilloscope and a strip chart recorder.

Microscopy. The fibre segments were viewed and photographed through a microscope, both at rest and during activation. A mercury vapour lamp or a high intensity flash source (Zeiss) was used for illumination, and the condenser (Zeiss model ZV) was stopped down to enhance

the contrast of the striations. High power photomicrographs were obtained using a 40× air objective (Zeiss, N.A. 0·65), a 10× eyepeiece (Zeiss) and Polaroid 107 film. These micrographs encompassed a 0·3 mm length of segment with a final magnification of about 460×. Low power photomicrographs were obtained using a 10× air objective (Zeiss, N.A. 0·22) and 5× eyepiece (Zeiss) and 35 mm film (Kodak High Contrast Copy Film). Segments up to 1·2 mm in length could be photographed in a single frame. The developed negatives were projected on an enlarger resulting in a final magnification of 110–350×, depending on the projection distance. The average sarcomere length within each frame was measured as the average spacing of three rows of striations running the entire length of the segment within the frame (Moss, 1979).

Protocol

The initial activation of each segment was done at a sarcomere length measured to be about 2·15 μm (varying from 2·10 to 2·20 μm) during steady contraction in solution of pCa 5·49. Segment length was then increased to give an estimated sarcomere length of 2·50, 2·75, 3·00 or 3·4 μm during contraction. Tension measurements and photomicrographs were obtained at two of the longer lengths before returning to the initial segment length for a control contraction. Photomicrographs obtained during activations of the fibre segments were taken when tension became maximal. At segment lengths greater than 1·2 mm, photomicrographs taken during two successive contractions were required to obtain an end-to-end, composite photograph of the segment. Tension measurements at an estimated sarcomere length of 3·4 μm or greater were always the last to be made on a given segment, since contractions at these lengths were often followed by a large decrease in the amount of tension developed at the control length.

Resting tension was measured by introducing slack into the segment and recording the change in the force baseline. For segment lengths at which there was significant tension in relaxing solution, active tension was measured as the tension developed in response to the activating solution. Tension values have been expressed relative to the tension (P_0) developed at the control length. In order to correct for fibre deterioration, the P_0 which pertained for each experimental tension measurement was determined by linear interpolation between the tensions obtained in control contractions at a sarcomere length of about 2·15 μm, just preceding and following the measurement. Segments were discarded if the tension obtained in the control contractions fell to 60% or less of that developed during the first activation of the segment.

RESULTS

Sarcomere length–tension relations during maximal calcium activation

The maximum tension developed by the fibre segments used in this study averaged 218 kN/m^2 in solutions of pCa 5·49 and at an average sarcomere length of about 2·15 μm. The force records in Fig. 2 show the variation of isometric tension in one segment as sarcomere length was varied. Length–tension data for two chemically and six mechanically skinned fibre segments activated in solutions of pCa 5·49 are plotted in Fig. 3, in which sarcomere lengths were measured from high power photomicrographs, as described in Methods. The tension developed by these preparations declined as sarcomere length was increased beyond about 2·15 μm, though the rate of decline was less than that observed by Gordon *et al.* (1966*a*, *b*) in tetanically stimulated, living muscle fibres. The amount of resting tension maintained by the relaxed segments, also shown in Fig. 3, was similar whether the segments were mechanically or chemically skinned. Resting tensions in the skinned segments were somewhat greater than would be expected from living fibres at similar sarcomere lengths (personal observations).

Length–tension measurements were done in eighteen additional fibre segments in which sarcomere length was measured from end-to-end photomicrographs of the

segments. Of these eighteen segments only eight had clearly visible striations along their entire lengths during maximal activation with calcium. Examples of the striation photographs which were obtained are shown in Pls. 1 and 2 for one of these eight fibre segments at two different stretched lengths. In this particular segment striation pattern uniformity apparently was maintained during maximal activations at long lengths. All of the length–tension data from the eight segments are plotted in

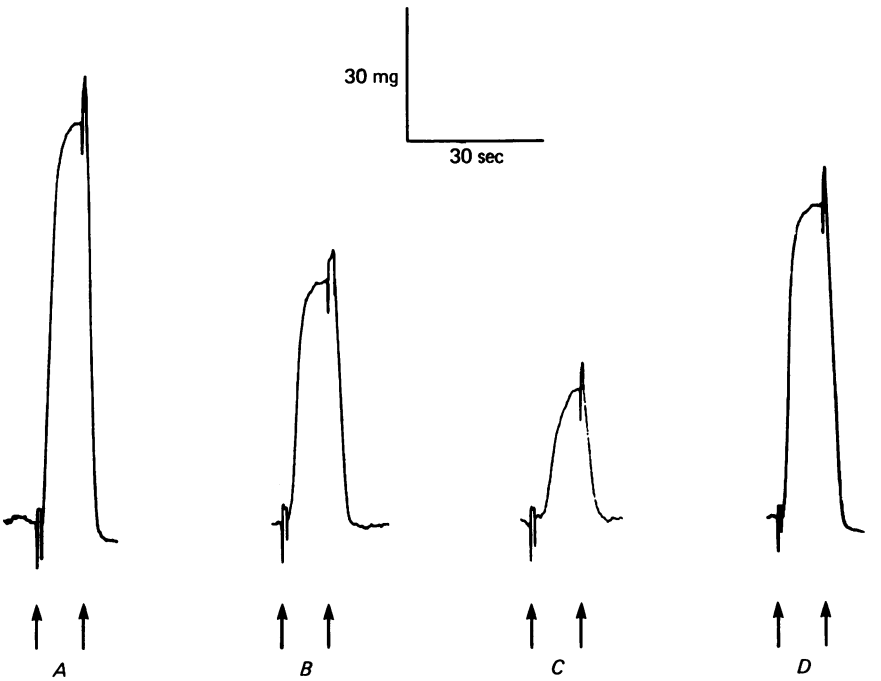

Fig. 2. Tension records obtained from a mechanically skinned fibre segment at pCa 5·49 and various lengths. The records were obtained in order from *A* to *D*. In each part, the first arrow indicates transfer of the segment from relaxing to activating solution, and the second arrow indicates the transfer back to relaxing solution. The average sarcomere lengths measured in the segments using high power photomicrographs were in part *A*, 2·20 μm; part *B*, 3·07 μm; part *C*, 3·40 μm, and part *D*, 2·14 μm. The relative tensions in parts *B* and *C* were 0·64 and 0·37 P_0, respectively. These values were corrected for fibre deterioration between the control contractions in parts *A* and *D*, as explained in Methods.

Fig. 4. Tension again declined as sarcomere length was increased, in agreement with the data of Fig. 3, though the data points from these segments generally lay closer to the relationship described by Gordon *et al.* (1966*a*, *b*). Data from segments in which striations were not visible from end-to-end were not included in Fig. 4, since in these cases an accurate determination of the average striation spacing was not possible.

Several investigators (Huxley & Peachey, 1961; Gordon *et al.* 1966*a*, *b*; Julian *et al.* 1978; Julian & Morgan, 1979) have presented evidence indicating that sarcomere length non-uniformity develops along a stretched living fibre during tetanic stimulation. In order to determine the degree of non-uniformity within our segments,

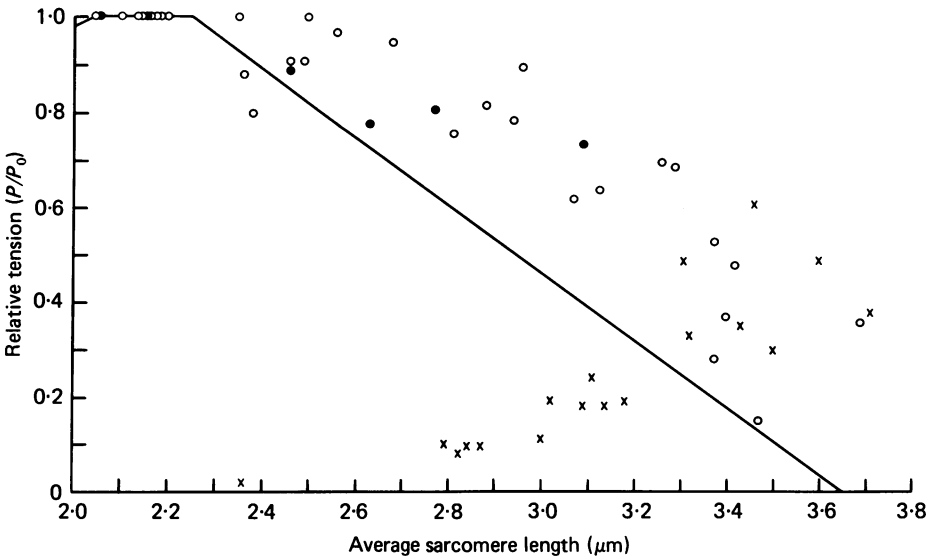

Fig. 3. Plot of passive (×) and active tensions *vs.* sarcomere length. Open circles are data from mechanically skinned and filled circles from chemically skinned fibre segments activated in solutions of pCa 5·49. Sarcomere length was measured from a 0·3 mm long central piece of each segment. Tensions are expressed relative to the tension developed by each segment in the range of sarcomere lengths between 2·10 and 2·20 μm. Continuous lines indicate the sarcomere length–tension relation described by Gordon *et al.* (1966*b*) in living, tetanically stimulated fibres.

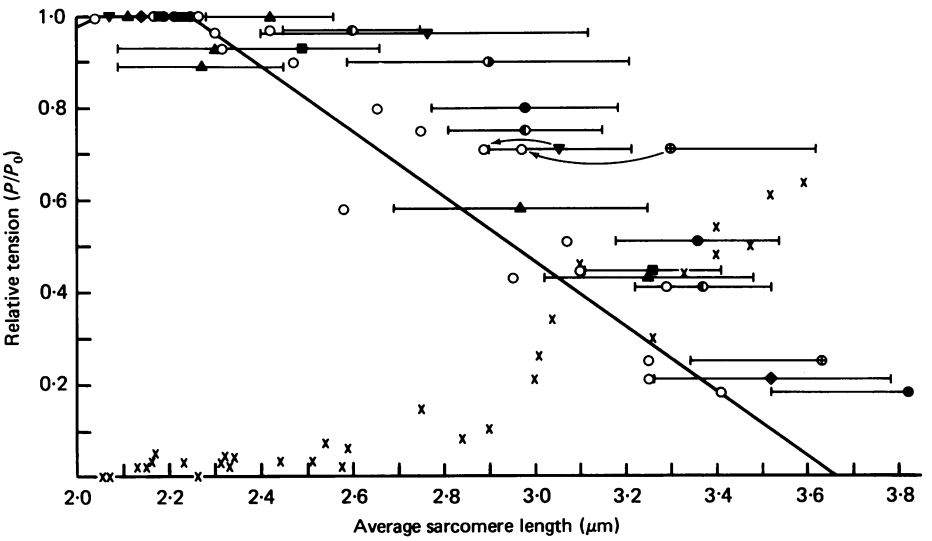

Fig. 4. Plot of passive (×) and active tensions *vs.* average sarcomere length, in solutions of pCa 5·49. The filled symbols, representing different fibre segments, are the tension data plotted against average sarcomere length measured from the whole of the fibre segment. The horizontal lines indicate ± 1 S.D. unit; in some cases, only one line is shown. The open circles are the tension data replotted against the shortest sarcomere length measured in a 50 sarcomere long piece of the segment. Relative tensions are expressed as in Fig. 3. The solid lines are from Gordon *et al.* (1966*b*).

the photomicrographs of the fibre segments were analysed to obtain plots of the average sarcomere length within portions of the segments versus the position of the portion from the centre of the segment. This was done by dividing the tracings of the photomicrographs into longitudinally adjoining portions, each fifty striations long, and then averaging the sarcomere length measurements from three parallel

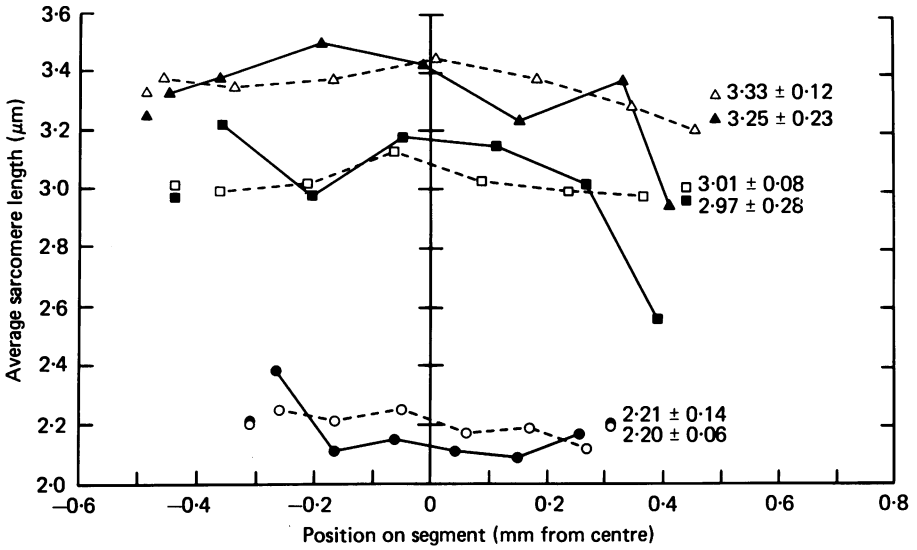

Fig. 5. Plots of the average length measured in fifty sarcomere long portions of fibre segment, *vs.* the position of each portion from the centre of the segment. Data was obtained from the chemically skinned segments represented as the filled, upright triangles in Fig. 3, at three over-all lengths: 0·62 mm (circles), 0·88 mm (squares), and 0·98 mm (triangles). In each case, the open symbols, connected by dashed lines, indicate passive sarcomere lengths; and the filled symbols, connected by continuous lines, indicate sarcomere lengths measured during steady force development in solutions of pCa 5·49. Each symbol is plotted in the centre position of the portion that the symbol represents. The portions at the ends of the segments in some instances had fewer than fifty sarcomeres. The symbols not joined by lines mark the ends of the segment, and also in each case indicate the average sarcomere length from the whole of the segment. The data in this Figure was obtained from the segment shown in Pls. 1 and 2. The force transducer end of the segment, as indicated in those Plates, is at the extreme right, i.e. positive, position in this Figure.

rows of striations within each portion. Examples of such plots for one of the fibre segments (that which is shown in Pls. 1 and 2 and also as the filled upright triangles in Fig. 4) at three different over-all lengths, both at rest and during contraction, are shown in Fig. 5. In each of the plots, it is apparent that during contraction, some regions of the segment shortened greatly at the expense of other regions while the over-all average sarcomere length changed little. (Note that with strings of fifty striations, the extremes in sarcomere length variation from the over-all average will be underestimated.) There was no tendency from segment to segment for the regions of relatively short or long sarcomeres to be located consistently at the ends or in the middles of the segments. At average sarcomere lengths greater than about 3·0 μm, the change in average sarcomere length upon activation was negligible,

presumably as a result of the increased resting tension having pulled taut the compliant ends of the segments within the connectors. Julian *et al.* (1978) have presented results which suggest that during fixed-end contractions of tetanically stimulated living fibres, the steady tension following the phase of tension creep most nearly represents the isometric tension of the shortest sarcomeres in the fibre. For this reason each of the tension values plotted against overall average sarcomere length in Fig. 4 has also been plotted against the shortest average sarcomere length which was measured from the fibre portions. The tensions plotted in this way agree well with the relationship described for tetanically stimulated living fibres during isometric contractions.

The remaining ten fibre segments, those having regions in which striations were grossly irregular during contraction, also yielded tension values during maximal activation which were higher than those expected on the basis of living fibre results. However, in these segments, correcting the sarcomere length to the shortest average length visible within a portion of the segment most often resulted in values which were still considerably above the length–tension relation for living fibres. An example of the striation pattern irregularities which were frequently observed during maximal activation at stretched lengths is shown for one fibre segment in Pl. 3. In this instance, the over-all average striation spacing was observed to increase from 2·84 μm while relaxed, to 2·98 μm during steady activation at pCa 5·49. The relative active tension was measured to be 0·80 P_0, which in a living fibre would be appropriate to a sarcomere length of about 2·5 μm. For such an increase in average sarcomere length to occur, undetected sarcomere shortening must certainly have taken place within the segment. The most likely places for this shortening to have occurred are the regions in which visible striations are lacking due presumably to misregistration of adjacent sarcomeres.

DISCUSSION

In solutions of pCa 5·49, the tension developed by the fibre segments of this study was found to decline as the average sarcomere length was increased to lengths greater than about 2·25 μm. However, when the over-all average sarcomere length was used to plot the tensions, the rate of decline of the present descending limb was less than that found by Gordon *et al.* (1966*a*, *b*) in living, tetanically stimulated fibres. A major difference between their study and our work is their use of a photo-electronic servo system to maintain a part of the fibre, selected for good sarcomere length uniformity, at a constant length during contraction. The use of the servo system minimized tension creep at long lengths (see also Julian *et al.* 1978). Gordon *et al.* (1966*a*) then used a technique of back extrapolation from the creep phase of the tension responses to obtain tension values at fibre lengths above the optimum. In fibres that are not under servo control creep is believed to occur as a result of the relatively short sarcomeres at the ends of the fibres shortening at the expense of the longer sarcomeres in the central portion of the fibre (Huxley & Peachey, 1961; Julian *et al.* 1978; Julian & Morgan, 1979). The fibre segments of the present study were held at constant over-all length, and because of their short lengths, no attempt was made to clamp the length of parts of the segments. Sarcomere length was instead measured from the whole of the segments.

In the plots of Fig. 5, it is evident that in the relaxed segments sarcomere length non-uniformities were present and that these were increased upon activation. The degree of non-uniformity, measured as the standard deviation of the sarcomere length measurements, increased as the average sarcomere length increased. Again from Fig. 5, the regions of shorter average sarcomere length apparently shortened against the regions of longer sarcomere length. When the tension developed by the segments at long length was instead plotted versus the shortest average sarcomere length measured from a piece of the segment (Fig. 4) there was substantially better agreement between the present data and that of Gordon *et al.* (1966*a*, *b*). This finding suggests that the active tension developed by the segments during contraction is most nearly a measure of the isometric tension-generating capability of the shortest sarcomeres in the segment. Presumably, the tension can be borne by the longer sarcomeres because of the presence in skinned fibres of relatively large passive tensions at moderately long sarcomere lengths. Also, if the longer sarcomeres are slowly stretched by the shorter sarcomeres, as has been shown to occur in living fibres (Huxley & Peachey, 1961; Julian *et al.* 1978), the sarcomeres being stretched would be able to maintain a tension greater than their isometric tension-generating capability due to the asymmetry of the force–velocity relation (see Katz, 1939).

Since this mechanism for extra active tension at long sarcomere lengths is similar to that proposed for creep (Julian *et al.* 1978), it is reasonable to expect that there would be a tension creep phase during the activation of the segments. Though creep-like phases were observed during some activations these did not occur consistently. The most likely explanation for the lack of creep in some contractions is that the time to develop full tension during an activation is several seconds longer than one would expect for the creep phase alone. The relatively slow rise of tension as the calcium-containing EGTA buffer system diffuses into the segment would tend to mask tension creep.

Our work shows that the measurement of the sarcomere length–tension relation in stretched skinned fibres is made difficult by the presence of sarcomere length non-uniformities in these preparations. Nevertheless, our data clearly show a descending limb above a sarcomere length of about 2·2 μm when tension is plotted against the over-all average sarcomere length measured from photomicrographs. There was no tendency for the length–tension relation to plateau (ter Keurs, Iwazumi & Pollack, 1978) or even to rise at long lengths as has been found by some investigators (Iwazumi & Pollack, 1979). When tension was plotted against the shortest average sarcomere length within a portion of the segment, there was good agreement between the present data and the descending limb obtained from living, tetanically stimulated muscle fibres (Gordon *et al.* 1966*a*, *b*). Plotting the data in this way can be justified on the basis of the well-known discontinuity in the force–velocity relation about the zero velocity point (Katz, 1939). The slope of the force–velocity relation is approximately 6 times greater for a frog muscle undergoing slow extension than for the same muscle while slowly shortening. Measurement of the shortest average sarcomere length therefore more nearly approximates the sarcomere length pertaining to the tension measured than does over-all average sarcomere length which would include significant weighting by sarcomeres undergoing slow stretch.

The use of photomicrography of the fibre segments in this study has been essential to the discovery of regions within the segments having severely disordered striations,

which in turn has helped to explain deviations from the descending limb first described by Gordon *et al.* (1966*a*, *b*). Even with photomicrographs some information about striation spacing is lost in that the parts of the segments above and below the plane of focus (approximately 20 μm thick) are not visible. Thus, photomicrographs provide information which while not complete is at least representative of the striation spacing along the segment. On the other hand, the use of laser diffraction to determine sarcomere length would result in measurements dominated by the ordered patches of striations within a segment, with little indication of greatly shortened or non-uniform regions. The use of this technique is further compromised by possible distorting effects of skewed striations on the diffraction pattern (Rüdel & Zite-Ferenczy, 1979).

The findings of the present study indicate that in skinned fibres at high and steady levels of activation the sarcomere length–tension relation at sarcomere lengths above about 2·05 μm is primarily determined by the amount of thick and thin filament overlap, as was previously concluded by Gordon *et al.* (1966*a*, *b*) for living fibres. The results of this study, and an earlier study involving the ascending limb (Moss, 1979), indicate that the form of the length–tension relation obtained in skinned fibres during high and steady calcium activation is similar to that obtained in living fibres during tetanic stimulation at low temperatures. This indicates that during tetanic stimulation at low temperatures the level of calcium activation in living fibres is relatively high and steady over the whole of the length–tension relation.

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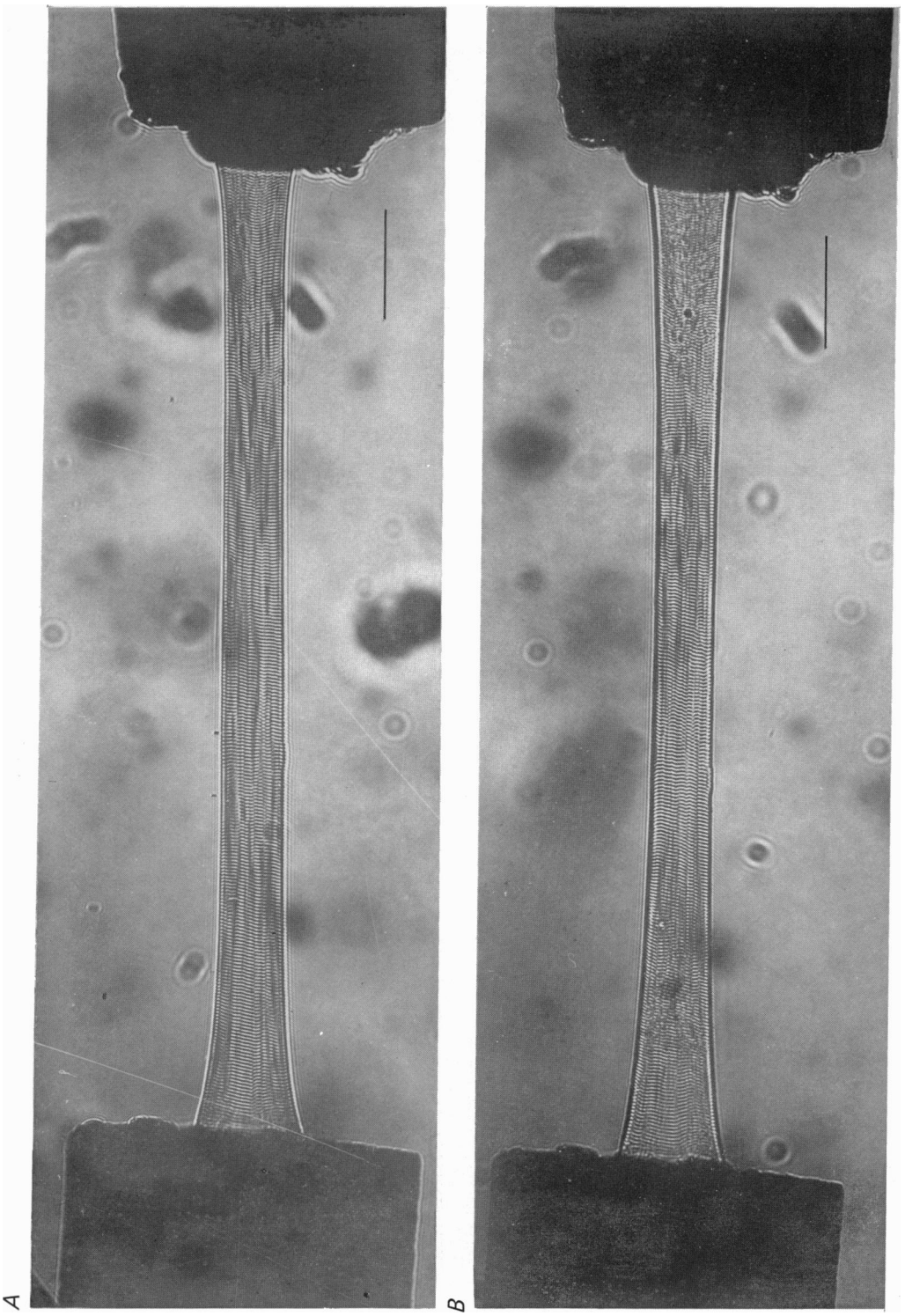
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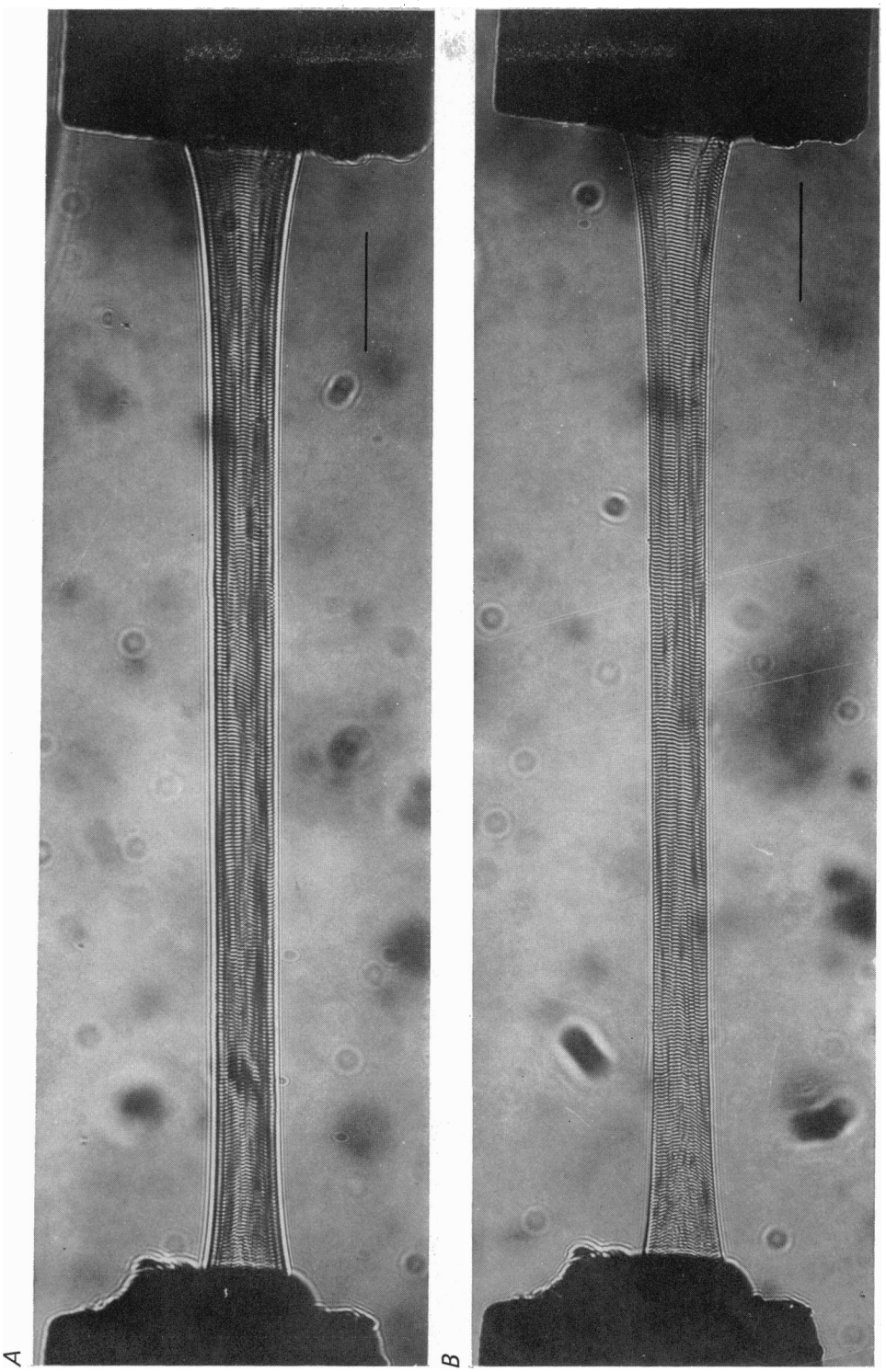
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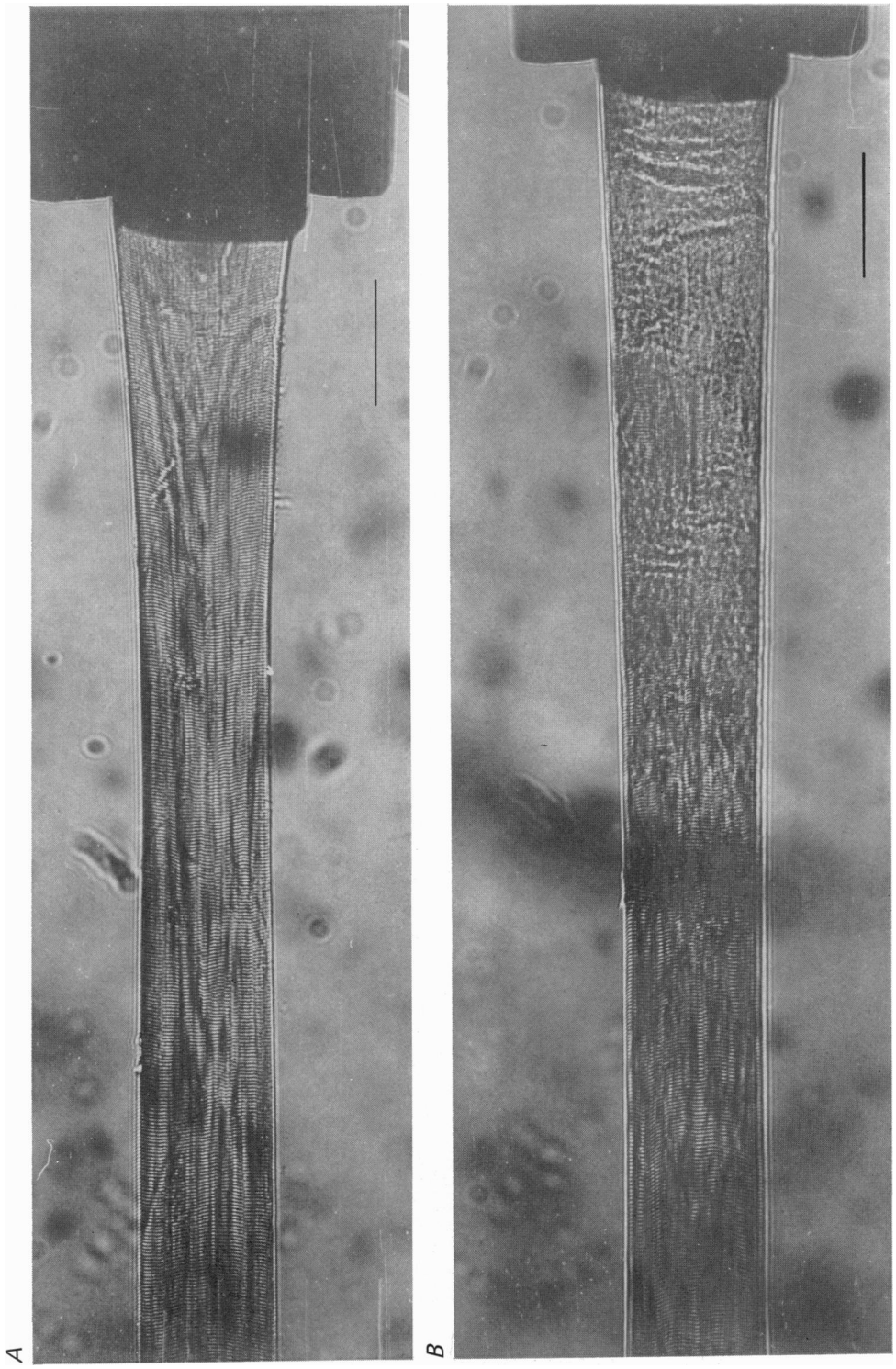
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EXPLANATION OF PLATES

Plate 1

Photomicrographs of a stretched, chemically skinned fibre segment while relaxed, part *A*, and during contraction, part *B*. Segment length is 0·88 mm. The over-all average sarcomere length was $3{\cdot}01 \pm 0{\cdot}08$ μm in part *A*, and $2{\cdot}97 \pm 0{\cdot}28$ μm in part *B*. The calibration bars on each photomicrograph indicate 100 μm. The relative active tension during the activation in *B* was 0·58. The force transducer end of the segment is uppermost in this micrograph.

Plate 2

Photomicrographs of stretched fibre segment while relaxed, in *A*, and during contraction in *B*. Segment is the same as that appearing in Pl. 1. Segment length is 0·98 mm. The over-all average sarcomere length was $3{\cdot}33 \pm 0{\cdot}12$ μm in *A*, and $3{\cdot}25 \pm 0{\cdot}23$ μm in *B*. The calibration bars indicate 100 μm. The relative active tension (P/P_0) during the activation in *B* was 0·43. The force transducer end of the segment is uppermost in this micrograph.

Plate 3

Photomicrographs of part of a chemically skinned fibre segment while relaxed, in *A*, and during contraction in solution of pCa 5·49, in *B*. The average sarcomere length was measured to be $2{\cdot}84 \pm 0{\cdot}07$ μm in a $2{\cdot}98 \pm 0{\cdot}17$ μm in *B*. Approximately one half of the fibre segment, the same portion in both cases, is shown in *A* and *B*. The relative tension developed during steady activation was $0{\cdot}80\ P_0$. Note in *B* that several regions of the segment lack visible striations. The calibration bars indicate 100 μm.